云 南 植 物 研 究 2009, 31 (3): 193～209
Acta Botanica Yunnanica DOI: 10.3724 SP.J.1143.2009.09086

外生菌根菌的分子生态学：分子标记基因、遗传个体及生态意义

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摘要：外生菌根真菌与很多植物形成互利共生关系，在营养物质交换和碳循环等方面起着关键性的作用，是森林生态系统的重要组成部分。近期生物技术的发展使得人们对外生菌根菌的群体遗传学和分子生态学有了更加深入的认识。本文介绍了一些常用的鉴定外生菌根菌的分子标记，并对每种分子标记的特点及其适用范围进行了讨论。文中总结了几种常用的鉴定未知外生菌根菌的方法，指出了一些在研究外生菌根菌过程中需要克服的内在困难，其中之一就是很多外生菌根菌不可以人工培养，所以人们缺少对其地下部分分布规律和动态变化的了解。在寄主专一性、物种多样性和丰富度、遗传个体大小、繁殖方式等方面，近期对外生菌根菌的分子生物学研究已经获得了很多重要的结果。作者讨论了这些研究成果对于今后开展外生菌根菌研究的重要意义以及在森林生态系统保育方面的潜在应用价值。

关键词：核糖体 RNA 基因；ITS；菌根；蘑菇；繁殖方式；生态相互作用
中图分类号：Q 948.12 **文献标识码**：A **文章编号**：0253 - 2700 (2009) 03 - 193 - 17

Molecular Ecology of Ectomycorrhizal Fungi: Molecular Markers, Genets and Ecological Importance

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Abstract: Ectomycorrhizal (EcM) fungi form mutualistic symbioses with many tree species and are regarded as key organisms involved in nutrient and carbon cycling in forest ecosystems. Recent technological advances have contributed significantly to our understanding of the population genetics and molecular ecology of EcM. In this review, we first present the commonly used molecular markers for characterizing individual EcM fungi. The properties of different types of molecular markers and their general utilities are discussed. We then summarize the common approaches for identifying unknown EcM fungi and point out the intrinsic difficulties associated with conducting EcM fungal research. One major deficiency is our lack of understanding of the below-ground distribution and dynamics of EcM fungi due to the non-cultivable nature of many EcM fungi. Recent molecular investigations of the EcM fungi have provided a variety of important data with regard to their host specificities, species diversity and abundance, genet size, and their reproductive strategies. We discuss the relevance of these findings to further functional investigations of EcM fungi and to potential implications in the conservation and management of forest ecosystems.

Key words: rRNA genes; ITS; Mycorrhizae; Mushroom; Mode of Reproduction; Ecological Interaction

Many of the known fungi are found with close associations of the roots of plant species, forming mutually beneficial symbiotic relationships. These fungi can colonize the plant roots and derive nutrients such as

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soluble carbohydrates, amino acids and vitamins from the plants . In return, plant hosts use the expanded surface of fungal mycelia to absorb water and minerals from soil (Read, 1991; Simard *et al.*, 1997, 2002; Table 1) . The fungi that colonize plant roots and form mutualistic relationships with their plant hosts are called mycorrhizae, a name derived from Greek that means " fungus roots ". In a mycorrhizal association, the fungus may colonize host roots either intracellularly or extracellularly, corresponding respectively to two groups of mycorrhizae: endomycorrhzae and ectomycorrhizae . In endomycorrhizae, fungal hyphae penetrate plant cell walls and form vesicles or arbuscules inside host plant cells . In contrast, in ectomycorrhizae, fungal hyphae grow extracellularly in the inter-cellular spaces and form sheaths around plant roots .

Table 1 Contrast between hosts and EcM fungi in terms of living benefits from each other

Plant hosts benefit from EcM fungi	EcM fungi benefit from its host
Supplying phosphorus, nitrogen, water	Supplying soluble carbohydrates
Protecting against pathogens	Providing niche space
Creating strong soil structure	Supplying amino acids
Facilitating nutrient transfer among plants	Ensuring ecological stability and evolutionary selectivity in nature
Enhancing cooperation and lowering competition among plants	Associating and interacting with other symbiotic microbes such as nitrogen fixers

The symbiotic association between plants and their mycorrhizae can be traced back to 400 - 460 million years ago, when the first plant appeared on land (Remy *et al.*, 1994) . Through the long history, both the plants and mycorrhizal fungi have adapted to benefit each other from the symbiosis . For example, many studies have demonstrated that mycorrhizal fungi play important roles for maintaining the normal growth of their plant partners (Jeffries *et al.*, 2003) . Without mycorrhizal colonization, plant hosts showed slower growth rate than those with EcM fungi (Smith and Read, 1997) . Recent research suggests that the reason for the poor performance of mycorrhizae-free plants could be due to their inadequate water and mineral uptake (Richard *et al.*, 2005; Selosse *et al.*, 2006) . Similarly, it has been demonstrated that plants with mycorrhizae are often more resistant to draught and to infectious diseases, such as those caused by microbial soil-borne pathogens (Bledsoe *et al.*, 1982) . However, for many plant-mycorrhizal associations, it still remains to be empirically determined whether this relationship is necessary and to what extent plants rely on fungi to maintain their normal growth in natural environments .

One significant issue in mycorrhizal research is the specificity of plant-mycorrhizal association . Some plants can form mycorrhizae with many different fungi while others with only a few . Similarly, some fungi can form mycorrhizae on many plant hosts while others with only one or a few . For example, trees such as oak, beech and birch form mycorrhizal relationships with only one or a few fungal partner (s) . *Lactarius deliciosus* is typically associated with *Pinus pinea* while *Suillus granulatus* and *Russula emetica* with *Pinus pinaster* (Gardes and Bruns, 1996a; Taylor and Bruns, 1999) . However, many photosynthetic plants seem to be able to form symbiosis with multiple unrelated EcM fungal species and many EcM fungi seem to be able to form symbiotic associations with multiple unrelated plants . Such broad associations provide significant potentials for mutual support among plants and fungi in natural ecosystems (Horton and Bruns, 2001) .

An estimated > 95% of all terrestrial plant species form mycorrhizal associations (Trappe, 1987) . The mycorrhizal fungi are broadly distributed across different phylogenetic groups and ecological niches . Such broad distributions are indicative of the importance of mycorrhizae in plant communities . As a result, mycorrhizal research has attracted a lot of attention from scientists in many disciplines such as mycology, plant biology, population biology, and ecology (Trappe, 1987) . The focus of this review will be on ectomycorrhizae .

There are several notable features about EcM fungi . First, EcM fungi are composed of many phylogenetically diverse species . More than 5, 000 fungal species have been estimated to form ectomycorrhizae worldwide (Amaranthus, 1998) . They include species from many genera, family, class and order in the phyla Ba-

sidiomycetes, Ascomycetes, and Zygomycetes (LePage *et al.*, 1997). Secondly, EcM fungi have board geographic ranges and are found in most regions of the global ecosystem. The main mycorrhizal centers of diversity and significant research are in temperate and tropical forests, such as those in southwestern China, northeastern China, the northwest pacific coast of North America, and northern Europe. Thirdly, EcM fungi contain many of the high-valued gourmet mushrooms such as truffles, matsutake, and chanterelles. Therefore, understanding their basic biology, systematics, population structures, and major reproductive strategies for EcM fungi can aid us develop better strategies to maintain their biodiversity and growth in natural ecosystems.

In contrast to ectomycorrhizal fungi, endomycorrhizal fungi fall into a single phyletic group, the Glomeromycota. The contrasting pattern and the polyphyletic nature of EcM fungi have raised many fundamental questions of EcM fungi themselves (Horton and Bruns, 2001), including (i) how diverse are EcM fungi in typical ecosystems? (ii) How many species are there and what is the most abundant species in EcM community? (iii) How specific are mycorrhizal fungi-plant symbiosis? (iv) What effects do EcM fungal populations exert on their local ecosystem? And (v) what is the history of EcM fungi? How have they evolved and diversified?

To address the above questions, both intrinsic and extrinsic difficulties need to be overcome with EcM community studies. Intrinsic hurdles come from EcM fungi themselves. First, as EcM fungi do not grow normally without their hosts, it is extremely difficult to simulate their growth in laboratory settings. Secondly, large numbers of underground EcM fungal species are still not described or identified. In early mycorrhizal research, only fruit bodies were used for study. This is because the vegetative structures of these fungi (mycorrhizae and mycelia) lying underneath the ground are small and morphologically inconspicuous, making it hard to obtain and distinguish. In addition, some EcM fungi do not produce fruiting bodies and they are rarely investigated (Horton and Bruns, 2001). Indeed, modern taxonomy of fungi was constructed mostly based on the analyses of fruiting structures, without genetic markers, matching species names to underground structures is problematic, and in certain instances, close to impossible. The multiple stages of EcM development also complicate research. Often, different stages and aspects of EcM fungi are investigated by different methods, and sometimes by different scientific disciplines. Some of the methods differentially used by different groups of scientists include morphological versus molecular methods in terms of identifying species, choices of different molecular markers, sensitivity in different molecular methods, dilemma in phylogenetic analyzing, etc. These challenges are discussed in the following text.

1 Characters and techniques used for studying EcM fungal communities

1.1 Morphology-visible characters for sorting EcM fungi

Morphological features are generally the first pieces of information we use for macro-fungi identifications. These features can be very easy to apply to and require little equipment and investment. Analysis of different morphotypes in the root tips can also be used to sort different groups of EcM fungi. This is because certain mycorrhizae contain signature attributes shared by a specific group (s) of fungi. However, more often than not, morphological features of mycorrizhae are ambiguous, especially among closely related species, making it hard to resolve unknowns to different species or even genera.

One common method to identify EcM fungi is to analyze morphological features of the fruiting bodies, relying on traditional fungal taxonomy that are based on characteristics of sexual reproductive structures. Commonly seen macroscopic characteristics of mycorrhizal fruiting bodies include size, shape, color of the cap, stem, flesh, gill, and concentric rings, etc; microscopic features include spore size, shape, and hyphae type, as well as the arrangements of spores and hyphae within the fruiting bodies etc. Consistent differences in these features have been used to identify unknowns, often down to the genus level. However, identification of

individual species usually requires more effort, since many morphological features are undistinguishable between closely related species . The presence of juices upon breaking, bruising reactions, spore prints are considered as additional methods to sort species as well as identify unknowns .

When conducting population level analyses, macroscopic morphological features are extremely useful for sorting distinctively different specimens . However, sometimes microscopic morphological sorting could become impractical when analyzing a large number of samples . In addition, if morphological analysis takes too long, the DNA in the samples may become degraded, resulting in failure in the subsequent molecular analyses . There are drawbacks in using morphological features alone for EcM fungi identification . Specifically, while macroscopic morphology can be used to sort fungi into discrete groups rapidly, it is often not sufficient enough to differentiate closely related species . Often, molecular analyses reveal multiple reproductively isolated cryptic species within morphological species (Bidochka *et al.*, 2005; Dettman *et al.*, 2003; Geml *et al.*, 2003; Kauserud *et al.*, 2006, 2007; Taylor *et al.*, 2000) . Furthermore, convergent evolution of certain trait features among unrelated species could lead to misidentification, when only morphological features are used .

Early mycologists developed many valuable morphological identification keys for fungal taxonomy . Some of these features were later compiled and analyzed for their usefulness . For example, in the analyses by Luoma *et al.*, 1997, 200 morphological types of ectomycorrhizal truffles and mushrooms from 189 soil cores were provided that included detailed descriptions for each morphotype (Luoma *et al.*, 1997) . Unfortunately, unknown samples' classification remains unclear due to a lack of report of their specific morphologies .

1.2 Molecular methods-fine scale sorting of EcM fungi

Due to the limitations in morphological analyses, molecular analyses have become increasingly common in fungal taxonomic and ecological research, including research into EcM fungi . In certain cases, morphotyping may be skipped entirely . The emerging field of metagenomics analyzes DNA samples directly from the environment, including both cultivable and un-cultivable ones (for a review in this area, see Xu 2006) . These developments are allowing us unprecedented access to microorganisms in nature . Below we describe and discuss some of the common molecular methods in EcM research .

1.2.1 Restriction Fragment Length Polymorphisms (RFLP)

RFLP is one of the most popular molecular methods to discriminate species or strains of fungi . It usually involves using restriction enzymes to digest genomic DNA, and analyzing the resulting patterns, with or without specific probes . Although sequencing can characterize DNA more thoroughly, RFLP analysis has been very popular, especially during the early years of applying molecular markers to fungal studies . Depending on the specific DNA fragment and restriction enzyme combination, RFLP can cluster unknown specimens into different groups, sometimes to the species level . The early success of RFLP includes low cost, fast and efficient . In whole genome digestion, RFLP cannot be used to detect polymorphisms for low copy number genetic elements . However, polymorphisms within high copy number genetic elements, for instance ribosomal DNA gene and mitochondrial DNA, can be detected using RFLP (Xu, 2005) . However, there are disadvantages associated with RFLP . For example, when the number of DNA bands is high, it is hard to distinguish two bands with similar migration abilities on the gel . In order to identify individual bands, specific labeled probes need to be used to recognize unknown bands through DNA-DNA hybridization (Xu, 2005) . Alternatively, specific DNA fragments can be amplified using highly selective primers through PCR and the PCR products can be then digested, and the resulting patterns compared side-by-side . Because of the highly conserved nature of the ribosomal RNA gene clusters, there is generally little or no difference among strains within the same species for this gene region . As a result, different restriction banding patterns for this gene region are usually indicative that the analyzed strains are of different species .

The main shortcoming of the RFLP typing is that when it is used alone, some samples cannot be successfully distinguished. In addition, there are several issues associated with RFLP pattern matching. Firstly, the databases are almost exclusively constructed using sporocarp samples but rarely nonsporocarp samples (i.e. in our case the EcM mycelia). The second problem is accuracy of RFLP pattern matching. For example, in the case where we could match a specific RFLP pattern to a species in the RFLP database, there might be some minor variations in fragment sizes that we fail to detect. Factors such as the choice of primers, the types of restriction enzymes, homogeneity of the gel, variation in electrical current can also affect the fragments' migrations through the gel matrix. Thirdly, RFLP is sensitive to intraspecific genetic variation due to single nucleotide polymorphism (SNP). While such polymorphisms can be useful for strain typing, they can also confound species identification in the absence of a robust database. Lastly, typical RFLP databases are limited in scope, often by the investigator's personal interests. Large improvement in many aspects such as increasing the sample size, standardizing the use of restriction enzymes, primers and intrinsic conditions of gel matrix to allow cross-lab comparisons. One of the most commonly used RFLP typing genomic region is the internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster. Both species and strain-specific ITS-RFLP patterns may be identified. As morphotyping and RFLP typing are becoming more inclusive, the integration of these two types of information could significantly enhance our ability for species and strain identifications.

1.2.2 PCR based molecular method——fast and accurate

To complement the shortcomings of the morphology-based approach and RFLP method, rapid PCR-based molecular analyses are often adopted and implemented in many fungal genetics research. PCR allows amplification of specific DNA fragments with either specific or non-specific primers that recognizes defined regions of the genomes (Mullis and Faloona, 1987). Because it requires very little genetic material and is typically gene specific in its amplification, the PCR methodology provides tremendous advantages over traditional molecular typing techniques. The large amount of products obtained in a typical PCR reaction allows scientists to conduct further analyses, including obtaining sequence information of the target genes. With sequence information in hand, many analyses can be performed including database searching and unknown-target mapping, sequence comparison and phylogenetic analysis. In EcM fungal community research, PCR has been used primarily for identification. However, other types of information, including the relative abundance of individual species are also possible from analyzing PCR products. For example, the ITS regions can be amplified through PCR from an ectomycorrhizal fungal community. The PCR products would contain a mixture of ITS sequences from different fungal species in the community. The PCR products can be directly sequenced using the pyrosequencing technique or cloned first into a host bacterium and then sequenced individually through conventional sequencing techniques. The obtained sequences can be analyzed that allow calculations of relative abundances of individual ITS sequence types (Xu, 2006).

1.2.3 DNA sequencing—the finest scale taxonomic identification

Sequencing one or several DNA markers is the most sensitive and robust way to identify species. There are many advantages of using DNA sequence based analysis for species and strain identifications. First, DNA sequences are unambiguous and some regions are highly conserved within species and can be used as molecular markers to characterize unknowns (Xu, 2005). Second, compared to morphological features, which represent individual phenotypes, DNA sequences make up genotypes at a very fine scale. Compared to pattern-based typing (RFLP, PCR fingerprinting), DNA sequence based typing can give more accurate and robust results (Xu, 2005). Third, DNA sequences can be stored in and retrieved from public databases. Such databases can serve as valuable resources for searching and editing (Xu, 2005). It also allows us to do fine scale genome typing, for example, intraspecific strain typing. Indeed, DNA-sequence based approaches are the future of EcM research.

1.2.4 ITS—an excellent molecular marker to identify species

The ITS region is a section of DNA located within the nuclear ribosomal RNA gene cluster (Fig.1). Each unit of the gene cluster comprises one small subunit 18S rRNA, a large subunit 28S rRNA, and a 5.8 S rRNA flanked by two non-coding DNA sequences known as ITS1 and ITS2 on either side. The combined length of ITS regions (including the 5.8S rRNA) in fungi is typically between 650 - 900 bp. Within an individual cell, there may be 50 - 200 copies of this unit cluster, linked by an intergenic sequence (IGS) that can be highly variable in length and sequence composition among species.

During the past two decades, the ITS region has become the most commonly used molecular marker in fungal ecology and systematic research (Egger *et al.*, 1995; Chambers *et al.*, 1998; McKendrick *et al.*, 2000). Its utility and popularity are due to the following reasons. First, it is present within the high copy number ribosomal RNA gene cluster as tandem repeats. As a result, a small number of cells may be sufficient for PCR and subsequent analyses. Second, it is highly conserved within species but can be highly variable between closely related species. Third, the ITS regions are relatively short and flanked by highly conserved sequences. Consequently, conserved primers applicable to broad groups of species can easily be designed and used to amplify the ITS regions by PCR. Two primers, ITS1 and ITS4, have been used widely in fungal systematics and population genetic studies. These two primers were originally designed from plant sequences and are used as universal primers across all fungi (White *et al.*, 1990, Gardes *et al.*, 1991).

Aside from ITS1 and ITS4, other taxa-specific primers have also been developed. For example, ITS1f and ITS4 are fungal specific; ITS1f and ITS4b are basidiomycete specific (Gardes and Bruns, 1993). These group specific primers are desirable in terms of enhancing specificity of one group and discriminating against other fungal groups. For instance, primer ITS4b has been designed to amplify sequences from all known

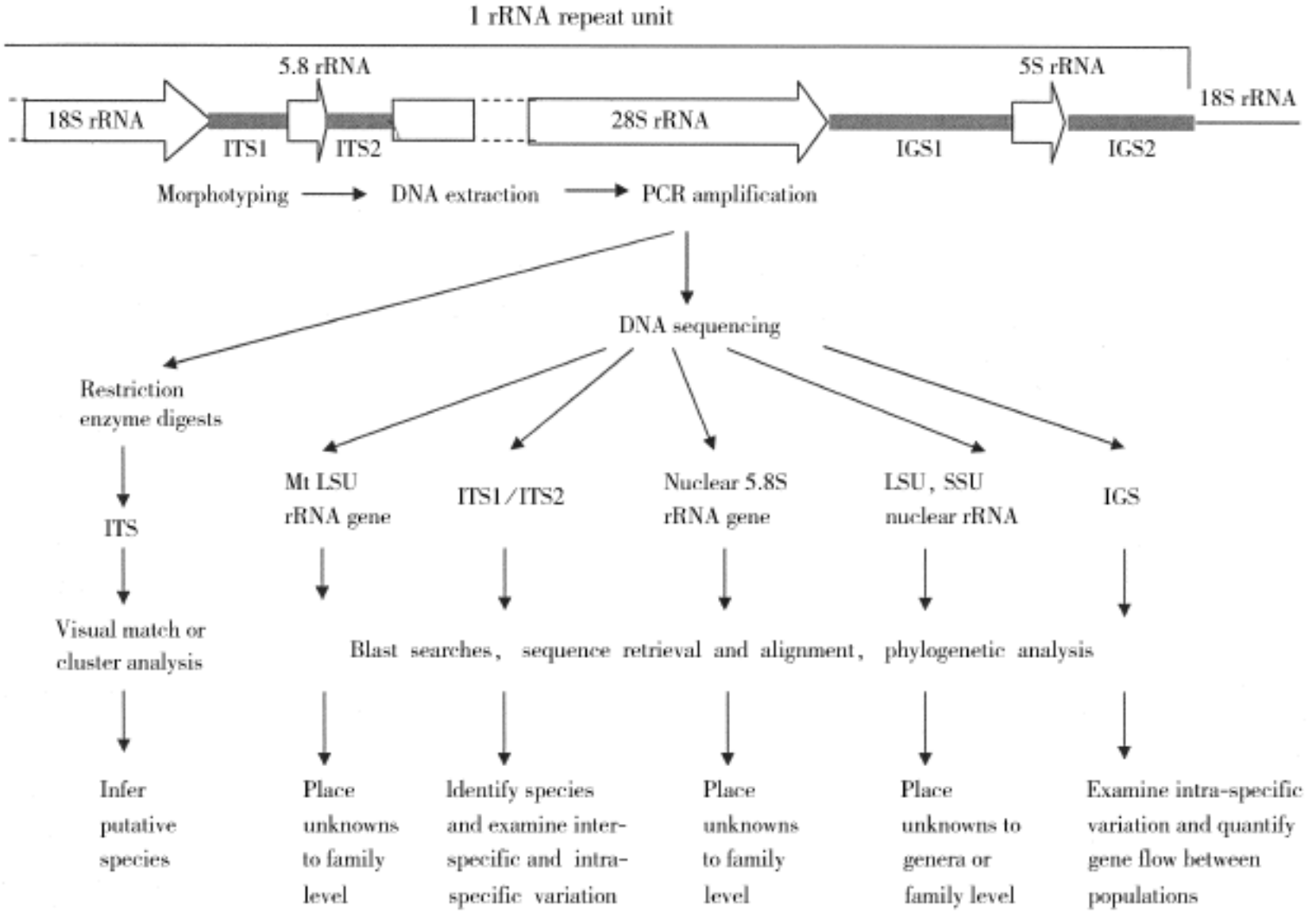

Fig. 1 A combined approach to identify unknown EcM fungi using ribosomal RNA genes to different taxonomic levels

basidiomycetes, while excluding fungi outside the group (Taylor *et al.*, 1999). Nevertheless, even though ITS1f ITS4b are specifically designed ITS primers for basidiomycetes, we do not expect that they will amplify every species within this group. This is because mutations could be present at the primer annealing sites for some potentially unknown basidiomycetes in nature. Indeed, non-amplified individuals with the above primers have been identified in previous experiments using other techniques. *Rhizoctonia* is one of the groups. Some species in the *Rhizoctonia* group cannot be amplified by the ITS1f ITS4b primer pair, even though it belongs to basidiomycetes (Robinson *et al.*, 2009). Indeed, due to their unknown nature in our databases, group-specific primers based on known taxa in that group will always have its limitations when working with unknown samples. One way to overcome the difficulty faced by group-specific primers is to use universal primers such as ITS1 ITS4 to reanalyze non-amplified species and to confirm the results. Bearing these caveats in mind, it's important to realize that care should be taken when interpreting the results.

The ITS region is currently the most popular DNA marker used in RFLP, especially for studying EcM fungal diversity. Matching the polymorphisms at the ITS regions has successfully separated many EcM fungal species (Egger *et al.*, 1995, Kårén *et al.*, 1997; Pritsch *et al.*, 1997). Indeed, ITS-RFLP has turned out to be the most convenient molecular method to sort fungal species with minimal technical requirements. As mentioned above, there are several advantages associated with ITS. In combination with PCR and RFLP, ITS PCR-RFLP has allowed generating species-specific banding patterns and making it possible to classify EcM fungi at the species level. This is because the ITS region usually contains restriction sites where common restriction enzymes could target. Commonly at least 2 to 3 frequent cutting restriction enzymes are needed for species identification. Since sequence differences between species can be due to both insertions deletions and nucleotide substitutions, different restriction enzymes may produce very different patterns. If several ITS PCR-RFLP patterns are associated with a single morphotype, additional samples with the same morphortype need to be selected and verified by RFLP. If such patterns are confirmed, taxonomic revisions may be necessary.

Nowadays, sequencing ITS regions has been routinely used to identify fungal species, and to study genetic diversity among different strains within one species. The ITS region is now perhaps the most widely sequenced DNA region in fungi (Bruns *et al.*, 1998; Pritsch *et al.*, 2000; Sha *et al.*, 2008; Xu, 2005). A large number of ITS sequences have been stored in GenBank. It has become a valuable resource for identifying unknown fungal species and for investigating the evolutionary relationships among fungal species (Bruns *et al.*, 1998; See also below for the UNITE database). Because of these and the previously mentioned features, the ITS regions have been adopted by the international mycological community as the barcode region for fungal identifications.

Other than the ITS sequences, additional molecular markers such as LSU rRNA, mtLSU, SSU rRNA, 5.8S nuclear rRNA are also widely used to discriminate fungal groups (Table 2). Due to the difference in the degree of resolution, unknowns can be placed into different taxonomic levels using different rRNA gene fragments (Table 2). The choice of which marker to use depends on the experimental goal, feasibility of PCR amplification of each region, and the availability of the sequence database of that particular marker for comparison and analyses. In many investigations, fine scale species and strain identifications are the final objectives. However, sometimes tracing the specimens down to family levels can provide sufficient information to answer certain phylogenetic questions. Species and strain-level identifications often require large databases (large taxa sets and multiple genes) to compare. Sometimes, even with large databases, there might be no matching with those in databases. Indeed, it is not uncommon that the ITS-based molecular identification can only reach the genus level (Gardes and Bruns, 1996b; Ciardo *et al.*, 2006).

When considering markers in addressing specific research questions, we need to realize that there is always a trade-off between cost and effectiveness in the

Table 2 Comparison of molecular markers within the ribosomal RNA gene cluster used for identification of unknown EcM fungi

	Advantage	Disadvantage	Popularity
SSU rRNA	Most conserved, excellent for high level taxonomic investigations	Too conserved for low taxonomic level investigations	Heavily used for identifying bacterial and archaeal diversity, but rarely for studying EcM fungi
LSU rRNA	More variable than SSU rRNA, can place to generic level	Database is limited	Few EcM studies used this gene fragment
5.8S rRNA	Very conserved, but can help resolve to phylum level	Sequences too short	Usually co-analyzed with ITS sequences
mtLSU rRNA	Somewhat variable, can help place to family level	Can have introns	Some studies used this gene fragment
ITS	Highly variable, extensive database, can place sample to species level	Too variable for high level taxonomic analyses and sometimes sequence alignment is difficult	Extensively used and a large database already available

type and number of markers selected for investigating certain issues . Relatively sensitive markers such as the ITS region works well for identification to the species level . However, for genotype-specific fine-scale identifications, other types of markers (e.g . single copy genes or inter-genic regions) are needed . With the increasing availability of candidate molecular markers, the best-fit candidate marker (s) may require prior screening for individual species before we can determine their appropriateness . Conversely, a less sensitive marker (e.g . the SSU rRNA gene) does a cruder sorting, but mapping and identification steps are easier and more accurate, since fewer samples may be needed to take into account . Another shortcoming for the ITS region is its high copy number, which makes it hard to distinguish whether heterozygosity is resulted by different copies of ITS on one chromosome or on two different chromosomes (in diploid or dikaryotic individuals) . Therefore it is hard to identify genotypes for individual alleles when the unknown is heterozygous .

1.2.5 Single-gene based phylogeny may not truly reveal organism phylogeny

The main drawback of single-gene typing is that it may not truly represent organism′s phylogeny (Doyle, 1992; Maddison, 1997) . For example, using a single gene as molecular marker to study organism phylogeny can be highly biased, since the gene chosen for analysis may evolve fast in some groups but relatively slow in other groups . Two species mapped together by a single marker could be actually very different from each other if analyzed with other genes . Simply speaking, each single gene has its intrinsic evolutionary bias . One way to minimize the bias is to use multiple molecular markers . Using multiple genes to study phylogeny is always more reliable than using only one gene since it is more representative for the entire genome (Xu *et al.*, 2006) .

2 Common approaches for examining species diversity within and among EcM communities

If the research focus was to examine the diversity of EcM fungi within a selected geographical area, combining morphology and PCR-RFLP of the ITS region should initially provide a rough grouping among a large number of fungal isolates . For individuals with nearly identical PCR-RFLP patterns, they can be further characterized by sequencing the ITS or other particular DNA markers .

If the research aim was to study one type of EcM fungi within a selected area, morphology or PCR-RFLP of the ITS region can be used as an initial step to identify the target EcM fungi . Further identification and confirmation can be achieved by PCR amplification and sequencing of marker gene fragments at other loci . After obtaining the sequence information, blast search can be carried out in order to determine the sequence identity with known specimens in databases . If the unknown specimens have high sequence similarities (usually > 97% for ITS regions) to sequences of known species deposited in the GenBank, the identity of the

unknowns can be inferred . However, if a high degree of sequence identity is not found, individual sequences are then used as a query to retrieve closely related sequences with comparable length from the GenBank or other publicly available databases . Representative sequences for each closely related species from GenBank were then included to compare potential intra-specific variation within different phylogenetic groups to known species . These sequences and all the retrieved sequences were then aligned using appropriate computer softwares . Subsequently, phylogenetic analyses applying different algorisms are used to reveal the phylogenetic relationship among the species . Usually, the most closely related known species are used as outgroups for references . Sometimes unknown fungi cannot be easily identified by examining a single gene . Sequence information of additional molecular markers could be helpful to identify unknowns . Since different genes evolve at different rates, multiple gene genealogy can limit the biases created by single genes and reveal the true phylogenetic relationships among species . In many cases, the relationships among strains and populations within species can also be revealed using sequences from multiple genes . Such sequence information can be used to determine the potential mode of reproduction and geographic patterns of molecular variations (e.g . Lan and Xu, 2006) .

While GenBank, DNA Data Bank of Japan (DDBJ), and the European Molecular Biology Library (EMBL) databases contain almost all publicly deposited DNA sequences that are cross-referenced with each other through unique accession numbers, the ectomycorrhizal research community has been benefited tremendously by another database called UNITE (http: unite.ut.ee) . UNITE is an rDNA sequence database focused specifically on ectomycorrhizal fungi in two phyla, the ascomycetes and the basidiomycetes . The database was established because of widespread sequence misidentifications and inaccurate reporting in GenBank, DDBJ and EMBL . To establish an accurate and reliable database, sequences in the UNITE database are generated from fruit bodies collected and identified by specialists and deposited in public herbaria that can be accessed by others . In addition, type specimens are used whenever possible . As of April 2009, the UNITE database contains 112363 fungal ITS sequences . Among these, 2736 were barcoding sequences from 1202 species in 128 genera . Aside from providing a robust database for identification of sequences from curated specimens, UNITE also has other features that facilitate the identification of fungal DNA directly from environmental sources (i.e . DNA sequences without specific specimens attached) . This search tool is becoming increasingly important because direct metagenomic analysis of environmental DNA is becoming increasingly common in EcM research .

2.1 Bioinformatic analyses of EcM fungi

Based on the aligned sequences, the relationships among sequences, strains, and or species can be revealed . The most common form of presentation for such relationships is through phylogenetic trees . Phylogenetic trees can be constructed using various algorisms implemented in different phylogenetic softwares . The most commonly used ones are maximum parsimony, neighbor-joining, maximum likelihood and Bayesian approaches . The trees generated by applying different algorisms are usually consistent with each other . However, in certain cases, they can be different from each other . The different patterns can be generated for a couple of reasons . One is the weighting scheme of polymorphic nucleotides . For example, the weights of transitional substitutions and transversional ones can have a significant effect on the final outcome of the analyses . The same can be said about insertions and deletions . Indeed, dozens of weighting methods have been developed to try to reflect the relative importance of different types of mutations during the evolution of specific lineages .

The second reason for different tree topologies generated by different methods using the same dataset relate to the differences in algorithms among the phylogenetic tree-construction methods . Different algorisms process data in different ways . The neighbor-joining method measures the genetic distance between each pair of taxa and joins taxa with the shortest distance first, followed by progressively more distantly-related

taxa (Saitou and Nei, 1987); maximum parsimony produces the most preferred phylogenetic tree invoking the fewest number of evolutionary changes (Felsenstein, 1978); maximum likelihood method searches for the tree with the highest probability or likelihood that matches the data (Fisher, 1978). Sometimes tree topologies are divergent at a great extent, making interpretation of phylogeny challenging. When such cases occur, the preliminary data can be used to generate specific hypotheses. Targeted additional sequence information can be then collected to test the hypotheses. In general, the greater the sample sizes and the more genes analyzed, the more robust the inferred evolutionary relationships among strains and species will be.

In conclusion, a combined morphological and molecular approach is generally used to address issues related to species diversity in EcM research. Morphological grouping is typically carried out at the initial stage of identification. Usually EcM fungi from each soil sample are classified into as many groups as possible based on their morphological characteristics. The morphological characterization can be then followed by ITS PCR-RFLP banding pattern matching with an established reference database. However, for fungi remaining unknown after comparison to PCR-RFLP database, DNA sequencing and phylogenetic analysis of ITS and various other molecular markers are needed. Consequently, the additional data can then be used to update the ITS-RFLP database. If more than one RFLP pattern is found associated with a single morphotype, additional samples with the same morphotype are further analyzed to exclude the possibilities of contamination and potential heterozygosity within individuals for the ITS regions (Horton and Bruns, 2001).

3 Common approaches for examining intraspecific variation among EcM fungi

Studies involving the examination of genetic variation within EcM fungal populations typically rely on multilocus polymorphisms using several types of markers, including single-copy gene based RFLP, PCR fingerprinting, DNA sequencing, and microsatellite DNA. These markers have been described in detail in an earlier review (e.g. Xu, 2005). Here, we briefly mention microsatellite markers. Microsatellite markers are those with variation in the number of simple sequence repeats within a DNA fragment that can be found among individuals within species. Because of their repetitive nature, they typically mutate much faster than single nucleotide substitutions, due to high frequency slippage during DNA replication. The high variability of microsatellite DNA makes them excellent molecular makers for strain typing and population analysis, especially for recently evolved populations. Together with the increasingly popular single nucleotide polymorphism, microsatellite DNA are helping scientists working on EcM fungi to determine species boundary, population structure, and reproductive strategy etc. Below we review recent studies on one specific issue, that of genet size of EcM fungi, in natural environments.

3.1 Genet-size and distribution

A genet is a group of individuals (fruiting bodies and or underground mycelia for EcM fungi) produced from one mating event and that occupy the same geographic area (Dahlberg and Stenlid, 1994; Xu, 2005). Bearing a small number of mutations, the individuals within a genet should be all genetically identical. Because they arise vegetatively from a single mating event, this shared descent among the individual fruiting bodies and mycelia implies connectivity both nutritionally and or genetically. Nutritional connectivity refers to the nutrients absorbed by mycelia from one location of the genet can be transported to another parts of the genet. Similarly, genetic connectivity means that mutations and horizontally transferred genes obtained in one area of the genet can be spread to other parts of the genet. At present, though neither connectivity has been demonstrated conclusively in nature, the existence of genets of various sizes suggests their likely importance in nature.

Significant research activities have been devoted to characterize the size of genets in EcM fungi. A variety of molecular markers mentioned above have been used to determine whether individual fruiting bodies or mycelia of one species from a defined geographic area

are genetically identical with each other but different from other individuals within the general region. Due to their high mutation rates, the polymorphisms at multiple microsatellite markers have been used extensively to robustly identify the size of individual genets. Typically, a single genet is identified by a unique microsatellite polymorphic type for individuals sampled at a defined geographic area. Similarly, genetic identity inferred using other types of markers such as random amplified polymorphic DNA (RAPD) is also widely used, especially during the early phase of ectomycorrhizal research.

The current data suggest that the size of genets vary widely among fungi. Small genets in species of the ectomycorrhizal genus *Russula* can be less than half a meter. In contrast, large genets have been found among plant pathogenic basidiomycete genus *Armillaria*. For example, in *A. gallica*, some genets can occupy up to 2, 200 acres (15 ha). For many fungi, sporocarps belonging to one genet usually are spatially arranged as a ring, known as a fairy ring. The fairy ring structure is generated from a single mating event. As mycelia grow outwards, the genet expands. Furthermore, if the spatial environmental conditions are relatively homogeneous, the genet expands at similar rates in all directions across the terrain. At certain times of the year when conditions favorable for mushroom fruiting appear, fruiting bodies are produced along the edge of the mycelial growth, forming a ring structure. Because mycelial growth depletes nutrients in the center of the ring, the size of the ring also expands over time. One striking feature of EcM fairy rings is the low species richness along the rings, dominated by the ring-forming species. However, fungal diversity can be high on both sides of the rings, with similar species diversities and compositions (Lian *et al.*, 2006; Hirose *et al.*, 2004). This fact suggests that EcM communities are capable of recovering soon after genet passage. However, questions such as how recovery is established soon after passage and how the dominance is achieved by the ring-forming species are still unclear.

Current molecular ecological investigations suggest significant variation among EcM fungi in their genet sizes. Table 3 summarizes the genet sizes of recently investigated EcM basidiomycete fungi. Genet size is typically measured in the largest distance between sporocarps that are produced by one mating event. As shown in Table 3, genet sizes vary greatly among species. *Laccaria* spp. and *Russula brevipes* have so far the smallest identified genets of less than 1 m in diameter (Baar *et al.*, 1994; Bergemann and Miller, 2002; Selosse *et al.*, 1999). Genets of *Tricholoma matsutake* and *Suillus grevillei* are less than 3 m in diameter (Lian *et al.*, 2006; Zhou *et al.*, 2001). Genet sizes

Table 3 Comparison of genet properties among EcM species. Genet size is measured based on the above-ground fruit body sampling. "-" indicates missing data

EcM species	Genet size	Estimated expansion rates (cm year)	Early or late stage?	References
Laccaria amethystina	< 1 m^2	60-110	Early	Gherbi *et al.*, 1999; Selosse *et al.*, 1999
Laccaria bicolor	< 12.5 m^2	220-100	Early	Baar *et al.*, 1994; Selosse *et al.*, 1999
Pisolithus tinctorius	< 40 m	-	Early	Anderson *et al.*, 1998
Hebeloma cylindrosporum	< 3.5 m	52.5	Early	Gryta *et al.*, 1997, 2000; Guidot *et al.*, 2001
Suillus pictus	3.4-21 m	50	Both	Hirose *et al.*, 2004
Suillus variegatus	29 m		Both	Dahlberg, 1997
Suillus pungens	40 m (maximum 300 m^2)	50	Both	Bonello *et al.*, 1998
Suillus grevillei	3 m	-	Both	Zhou *et al.*, 1999
Suillus bovinu	3-30 m	-	Both	Dahlberg and Stenlid, 1990
Russula brevipes	< 3 m	-	Late	Bergemann and Miller, 2002
Russula cremoricolor	0.38-1.27 m	-	Late	Redecker *et al.*, 2001
Russula vinosa	< 1 m	-	Late	Liang *et al.*, 2004b
Amanita francheti	1.5 m^2	-	Late	Redecker *et al.*, 2001
Cortinarius rotundisporus	30 m	-	Late	Sawyer *et al.*, 1999
Cantharellus formosus	2-13 m	-	Late	Dunham *et al.*, 2003
Lactarius xanthogalactus	9.3 m^2	-	Late	Redecker *et al.*, 2001
Tricholoma matsutake	2 m	10.3	Late	Lian *et al.*, 2006

greater than 20 m are found in several species of the genus *Suillus* (Hirose *et al.*, 2004) . To estimate genet expansion rate, spatial distribution of sporocarps within the same genet is typically monitored through several years . The results from previous studies show that genet expansion rates also vary among species . For example, *Tricholoma matsutake* expands relatively slowly with an average rate of 10.3 cm yr, while *Hebeloma cylindrosporum* has a much higher rate of 45 - 60 cm yr (Gryta *et al.*, 2000; Lian *et al.*, 2006) . Some of *Laccaria* spp . genets have extremely high expansion rates, around 100 cm yr (Gryta *et al.*, 1997, 2000; Bonello *et al.*, 1998; Guidot *et al.*, 2001) . Taken together, these estimates suggest that EcM genets can live up to about 30 - 40 years in natural ecosystems .

3.2 Early-stage versus late-stage EcM fungi

Typical natural ecological communities, including the EcM communities, are established through a series of changes of biological compositions and abiotic factors . The process of ecological change involving a series of natural communities that are established and replaced over time is called a succession . There are two kinds of ecological successions, primary succession and secondary succession . Primary succession occurs in an environment in which new substrates, devoid of any living organism and usually lacking soil, is deposited (for example a lava flow) and allows the establishment of an ecological community . In primary succession, pioneer species like mosses, lichen, algae and fungi first colonize the substrate . Together with abiotic factors such as wind, water and the heat cold cycle, these pioneer species change the substrates, making them suitable for subsequent growth of plants . The plants then dominate but often replaced successively by plants better adapted to less austere conditions . Examples of primary succession can be found on a new lava flow, an area left from retreated glacier, or abandoned strip mine .

In contrast, secondary succession is a response to a disturbance, for example, forest fire, tsunami, hurricane, flood, or an abandoned field . While primary succession takes place in an area that is originally without any living organism, secondary succession occurs in an area where life once existed but has since been destroyed or disturbed, for example by fire, tornado, harvesting or other human activities such as agriculture, that reduces an already established ecosystem (e.g . a forest or a wheat field) to a smaller population of species . As such, secondary succession occurs on preexisting soil, unlike primary succession that usually occurs in a place lacking soil .

EcM fungi can be found associated with both primary and secondary successions . EcM fungi associated with primary succession plants are called early-stage EcM fungi . Some typical early colonizing fungal species are *Hebeloma cylindrosporum* (Gryta *et al.*, 1997, 2000), *Laccaria bicolor* (Baar *et al.*, 1994) and *Pisolithus tinctorius* (Anderson *et al.*, 1998) . EcM fungi found in secondary successions are called late-stage EcM fungi . Typical EcM fungi in late-stage succession are those in families Russulaceae, Cortinariaceae and Amanitaceae . Early colonizing fungal species typically colonize by spores . Because primary succession niches are typically poor in nutrients these early EcM fungi are expected to have relatively small and non-persistent genets (Deacon *et al.*, 1983; Fox 1983) . Early-stage fungi are considered as R-selected species, because they produce many offspring and are capable of filling available niches in an environment very quickly (Deacon and Fleming, 1992) . In contrast, late-stage EcM fungi may also colonize initially by spores or by dormant mycelia underground, but because of nutrient availability from the soil, they are able to spread by mycelial growth and expand . As a result, their genets are expected to be large and temporally persistent (Dahlberg and Stenlid, 1990) . Late-stage EcM fungi may be considered K-selected species, more capable of dealing with environmental stresses (Cooke and Rayner, 1984; Grime *et al.*, 1979) . While the typical late-stage EcM fungi in genera *Russula*, *Amanita*, *Lactarius*, are expected to propagate by mycelial growth, researchers have found that their genet sizes and age vary significantly, suggesting that proliferation by sexual spores in these late-stage fungi might be more important than previously expected (Bergemann and Miller, 2002) . Even in mature plant communities with

relatively homogeneous ecological conditions, the genet sizes can vary significantly, suggesting reproduction by spores is a prominent feature of late-stage EcM fungi. Thus, the appearance of a species in secondary succession communities cannot be used to draw conclusions about their genet size and modes of reproduction and colonization (Bonello *et al.*, 1998).

3.3 Factors affecting the reproductive modes and persistence of mycelia network

The mode of reproduction for EcM fungi is typically inferred based on the associations of alleles at the same or different loci. Populations with alleles randomly associating with each other are considered sexually reproducing populations while those with significant signatures of non-random associations are considered asexual populations (Xu, 2005). Another indicator in EcM fungi about the relative importance of sexual and asexual reproduction is the size of genets. By examining the genet sizes of EcM fungal species in nature, the reproductive strategies of individual species may be predicted. It has been suggested that species forming many small genets likely reproduce mainly by spore colonization and the species that form large genets mainly propagate by underground mycelial extension (Anderson *et al.*, 1998; Bonello *et al.*, 1998; Dahlberg and Stenlid, 1990, 1994; Dahlberg, 1997; Zhou *et al.*, 1999). Most species use a combination of both strategies. For example, *Suillus* spp. use a mixed strategy: they form many small genets by spore colonization at the early stage of lifecycle, while then produce large genets by mycelia expansion at late stages (Dahlberg and Stenlid, 1990).

Studies of *Suillus* spp. also revealed that the size of genets is correlated with age of host stands (Dahlberg and Stenlid, 1994). Small genets are mainly found in young-aged stands, while large genets are found in mature stands. However, exceptions have been found in *Lactarius xanthogalctus* and *Russula cremoricolor*, which form many small genets in mature tree forests (Redecker *et al.*, 2001). The age of stands is thus not sufficient to predict colonization strategies across all EcM species. The spatial pattern of EcM fungi also depends on many other factors, such as (a) environmental physical parameters, e.g. temperature, moisture, and light; (b) edaphic factors, e.g. soil moisture, depth of organic matter, and soil pH (Erland and Taylor, 2002); (c) biological traits of the fungi; and (d) competition with other fungal species (Anderson *et al.*, 1998; Dahlberg and Stenlid, 1990, 1994; Dhlberg, 1997). Guidot et al. demonstrated that *Hebeloma cylindroporum* forms large genets under conditions with low competition with their neighbor fungi (Guidot *et al.*, 2001). Conversely, small genets are observed under high intensities of competition. The capacity to survive and expand for a long period of time in the soil as generative mycelia and the ability to colonize using spores differ greatly among species. In general, in the laboratory settings, fungi tend to reproduce sexually when environmental conditions are unfavorable, and reproduce asexually when environmental conditions become favorable. The fruiting seasons for many EcM mushrooms are associated with the onset of stressful environmental conditions, consistent with laboratory observations.

Genet expansion rates, also known as mycelial expansion rate vary among species in a great range from 10 cm yr to 110 cm yr (Table 3). The variation could be due to a combination of genetic and environmental factors, such as mentioned above (Bonello *et al.*, 1998; Lian *et al.*, 2006). In the studies of most EcM species, genet expansion rates are relatively constant within species through several years of growth (Bonello *et al.*, 1998; Dahlberg and Stenlid, 1994; Lian *et al.*, 2006; Zhou *et al.*, 1999). While EcM genet establishment and growth rates are known to be affected by environmental factors, however, the specific mechanisms remain largely unknown. Future research should be focusing on monitoring genet progression in various environments.

The ability of EcM mycelia to expand is a unique characteristic for filamentous fungi, therefore studying genet size can understand the ecological features of these species in nature. The characteristics of EcM genet can be used as indicators of succession stage of forests, individual population boundary, and the required area of study site in EcM research (Liang *et al.*, 2004a). It can also help understand the roles of

spore dispersal and mycelial growth in the life histories of EcM fungi . For example, as a late-stage EcM fungi, species in *Russula* maintain reproduction by sexual spores even in suitable environmental conditions with minimum competitors, indicating that spore colonization plays a much more important role in the life history of *Russula* even in mature forests (Redecker *et al.*, 2001) .

Though still preliminary, the data in Table 3 also suggest that phylogenetic background of species may play a role in genet size . Specifically, if the phylogenetic background were important, we should expect to see similar genet sizes among closely related species . Based on the limited data available, species in certain genera, e.g . *Suillus*, all seemed to have relatively large genets . However, this may not hold in other taxonomic groups . For example, distinct genet sizes and propagation strategies are often found among closely related taxa in other genera, e.g . between two sister species of *Rhizopogon* (Kretzer *et al.*, 2003; Table 3) . Further analyses of more taxa in representative environmental conditions are needed to critically test this hypothesis .

4 Ecological importance of EcM fungi diversity

Aside from genet size differences, EcM fungi can vary significantly in other aspects, including the substrates they inhabit, the ability to uptake various nutrients, and the tolerance to water stress and temperature extremes . Because different groups of EcM fungi have different adaptive features, we expect that plants associated with a greater diversity of EcM fungi in their roots to be fitter and more capable of surviving and reproducing . In contrast, plants associated with a single type of EcM fungi may be vulnerable to environmental stresses . However this hypothesis has not been tested either in laboratory or field conditions, therefore whether high diversity of colonizing fungi could enhance the survival of plants remains unclear .

Previous researches on EcM fungi community structure have demonstrated that the abundance and rarity in EcM community seemed to show reverse relationships (Horton and Bruns, 2001) . This pattern means that the EcM samples that we randomly select in nature are likely highly biased . The high number of rare EcM species could mask the actual abundance of common EcM species in a selected EcM community . The bigger the sample size, the more species and genotypes will likely be identified . Whether a sample size would be representative of the EcM community structure can be determined using saturation analysis . In this analysis, when the number of samples goes up, the number of species should also go up, to a point when it becomes stable (McCune and Mefford, 1999) . The number of samples before the curve reaches plateau is the suggested one that can robustly represent the EcM fungal diversity . Jackknife estimate can also be used to predict the number of taxa needed to resolve the community structure (Mueller-Dombois and Ellenberg, 1974; Palmer *et al.*, 1991) .

Based on studies on EcM community conducted so far, the most dominant EcM fungi fruiting bodies are found belonging to Russulaceae, Thelephoraceae, and non-thelephoroid resupinates (Gardes and Bruns, 1996a; Jonsson *et al.*, 1999c; Luoma *et al.*, 1997) . At present, the reasons for their numerical and potentially functional dominance are unknown . However, it has been suggested that for most EcM fungi, the ones that fruit the most abundant are not necessarily the ones most abundant as mycorrhizae (Gardes and Bruns, 1996a; Jonsson *et al.*, 1999 a, b; Mehmann, 1995) . One possibility is that species that are abundant as mycorrhizae on roots but fruits infrequently likely require more energy for sexual reproduction than for vegetative growth . Direct analyses of metabolisms and energy consumptions are needed to test this hypothesis .

A better understanding the community structure and the relationship between hosts and symbiotic EcM fungi will allow us better able to develop sound strategies to conserve the diversity of EcM fungi . This in turn will help construct a suitable forest management practices to protect forest ecosystems . In these developments, two specific issues warrant consideration: (1) different EcM fungi play different roles in plant growth; and (2) different EcM communities are associated with

different aged forests (Molina and Trappe, 1982; Sylvia *et al.*, 1997). Often, diverse EcM fungi are found associated with mature trees, and this association can benefit nearby seedlings (Sylvia *et al.*, 1997). It has been demonstrated that a greater EcM diversity exists for seedlings located closer to mature trees than those closer to immature or newly-planted tress (Sylvia *et al.*, 1997). This result suggests that mature trees can be used as a reservoir to help maintain diverse EcM fungi for the broader forest community. Furthermore, Taylor *et al*. (1989) identified a high diversity of EcM fungi in the trees whose nearby trees are from other species. Therefore, planting a mixture of tree species can also be a great way to improve EcM diversity. In these mixed forest ecosystems, species with broad host ranges will likely be more competitive than those with narrow host ranges. Therefore, during or after timber harvesting, representative host plants should be retained and or replanted.

5 Concluding remarks and future directions

In this review, we outlined a set of methods for identifying EcM fungi and illustrated their utilities in EcM research on genet size, modes of reproduction, and ecology. A combination of morphological and DNA-based molecular techniques is often required to identify large numbers of unknown EcM fungi in natural ecosystems. Samples of both sporocarps and mycorrhizae are needed to help obtain a clear picture of the richness and abundance of EcM fungal species in a particular study site. At present, the full extent of EcM fungal diversity remains largely unknown. Future experiments should directly examine the relationships between EcM fungal diversity and function in representative ecosystems. In addition, the patterns and mechanisms for the specificity and inter-connectedness among different host plants, different mycorrhizal fungi, and between mycorrhizal fungi and plants will be of great interest for both basic scientific investigations and applied research. The molecular ecological analyses of more EcM fungi should help us understand the broad patterns of genet sizes and their modes of reproduction. Such understandings will help develop suitable forest management strategies.

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